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Published in:
Food and Chemical Toxicology

DOI:
[10.1016/j.fct.2020.111459](https://doi.org/10.1016/j.fct.2020.111459)

Publication date:
2020

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Document Version
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Schultrich, K., Henderson, C. J., Buhrke, T., & Braeuning, A. (2020). Effects of 2-MCPD on oxidative stress in different organs of male mice. *Food and Chemical Toxicology*, 142, [111459].
<https://doi.org/10.1016/j.fct.2020.111459>

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PII: S0278-6915(20)30349-5

DOI: <https://doi.org/10.1016/j.fct.2020.111459>

Reference: FCT 111459

To appear in: *Food and Chemical Toxicology*

Received Date: 21 April 2020

Revised Date: 25 May 2020

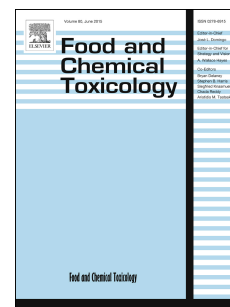
Accepted Date: 25 May 2020

Please cite this article as: Schultrich, K., Henderson, C.J., Buhrke, T., Braeuning, A., Effects of 2-MCPD on oxidative stress in different organs of male mice, *Food and Chemical Toxicology*, <https://doi.org/10.1016/j.fct.2020.111459>.

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Abstract

2-Monochloropropane-1,3-diol (2-MCPD) and its isomer 3-monochloropropane-1,2-diol (3-MCPD) are widespread food contaminants. 3-MCPD has been classified as a non-genotoxic carcinogen, whereas very limited toxicological data are available for 2-MCPD. Animal studies indicate that heart and skeletal muscle are target organs of 2-MCPD. Oxidative stress may play a role in this process, and the potential of 3-MCPD to induce oxidative stress *in vivo* has already been demonstrated. To investigate the potential of 2-MCPD to induce oxidative stress *in vivo*, a 28-day oral feeding study in male HOTT reporter mice was conducted. This mouse model allows monitoring substance-induced oxidative stress in various target organs on the basis of *Hmox1* promoter activation. Repeated daily doses of up to 100 mg 2-MCPD/kg body weight did not result in substantial toxicity. Furthermore, the highest dose of 2-MCPD had only minor effects on oxidative stress in kidney and testes, whereas brain, heart and skeletal muscle were not affected. Additionally, 2-MCPD caused only mild changes in the expression of Nrf2-dependent genes and only slightly affected the redox status of the redox-sensor protein DJ-1. Thus, the data indicate that 2-MCPD, in contrast to its isomer 3-MCPD, does not lead to a considerable induction of oxidative stress in male mice.

Effects of 2-MCPD on oxidative stress in different organs of male mice

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1. Introduction

Chlorinated propanols such as 2-monochloropropane-1,3-diol (2-MCPD) or 3-monochloropropane-1,2-diol (3-MCPD) and their fatty acid esters are widespread process contaminants in food. They are mainly formed as by-products when vegetable fats and oils are refined at high temperatures (Craft et al. 2013; Kuhlmann 2011). Especially palm fat and oil, which are added to many ready-to-eat processed foods, often contain high amounts of MCPD fatty acid esters (Weißhaar and Perz 2010). In vegetable oils, amounts of 3-MCPD fatty acid esters are approximately twice as high as of 2-MCPD fatty acid esters (EFSA 2016). After oral intake of MCPD fatty acid esters, they are largely hydrolyzed during the gastrointestinal passage, and free MCPD becomes bioavailable, which is then readily resorbed into the body (Abraham et al. 2013; Barocelli et al. 2011). In comparison to the isomeric 2-MCPD, the toxicity of 3-MCPD has been extensively investigated. In long-term animal studies, 3-MCPD induced neoplastic changes in kidneys and testes of rats, including Leydig cell and renal tubule adenomas as well as renal tubule carcinomas (Cho et al. 2008; Sunahara et al. 1993). These findings make both kidney and testis the main target organs of 3-MCPD toxicity. Accordingly, the International Agency for Research on Cancer (IARC) classified 3-MCPD as possible carcinogenic to humans (category 2B) (IARC 2012). Based on data from the animal studies, a tolerable daily intake (TDI) of 2 µg 3-MCPD per kg body weight was derived (EFSA 2018). This value may be exceeded in particular by infants which are exclusively fed with infant formula and follow-up formula as these formulas usually contain considerable amounts of palm oil (German Federal Institute for Risk Assessment 2007; German Federal Institute for Risk Assessment 2020).

Conversely, for 2-MCPD only very limited toxicological data are available, which are not yet sufficient for the derivation of a health-based guidance value. According to an EFSA report from 2016, a 28-day oral toxicity study with rats has revealed myocardium, skeletal muscle and kidneys as potential target organs for 2-MCPD toxicity (EFSA 2016). To understand the

molecular mechanisms of toxicity of 2-MCPD and 3-MCPD, proteomic and transcriptomic analyses have been conducted based on 28-day feeding studies with rats. These data showed that the toxicological profiles differ between 3-MCPD and 2-MCPD, and bioinformatic analyses of the omics data suggest that both substances might have an effect on oxidative stress-related processes (Buhrke et al. 2017; Frenzel et al. 2018a; Frenzel et al. 2018b; Schultrich et al. 2017). The results of a more detailed molecular study revealed that MCPD induces oxidation of a redox-sensitive cysteine residue in the stress-response protein DJ-1, and that oxidized DJ-1 may serve as multi-tissue effect marker for 3-MCPD-mediated toxicity. 2-MCPD, however, led to a substantially less pronounced oxidation of the thiol group of DJ-1/Park7 in rat, as compared to 3-MCPD (Buhrke et al. 2018), possibly indicating a lower toxic potential of 2-MCPD regarding the induction of oxidative stress. Moreover, an *in vivo* study with HOTT (heme oxygenase transgenic triple reporter) mice has demonstrated substantial effects of 3-MCPD in mice (Schultrich et al. 2020). The HOTT mouse serves as a model to monitor substance-induced oxidative stress in different target organs. The repeated exposure to 3-MCPD for 28 days resulted in a dose-dependent activation of the ROS-sensitive *Hmox1* promoter and an irreversible oxidation of the redox-active protein DJ-1 in kidney, testes and specific brain areas of the mouse.

The toxicity of 2-MCPD and the underlying mechanisms of action are barely known. As mentioned above, available histological data point towards potential target organs in rats, and omics analyses suggest a possible role of oxidative stress in 2-MCPD toxicity. The present study therefore aimed at gaining detailed knowledge on 2-MCPD-induced oxidative stress in different target organs on the basis of a 28-day oral feeding study with HOTT mice.

2. Materials and Methods

2.1 Animal study

The animal study was conducted by the Medical School Resource Unit of the University of Dundee in accordance with the Animals (Scientific Procedures) Act (1986) and EU Directive 2010/63/EU after receiving approval from the Welfare and Ethical Treatment Committee and the University Veterinary Surgeons. The heme oxygenase 1 transgenic triple reporter (HOTT) mouse, on a C57BL/6NTac background, has a heterozygous genotype and has been developed, described and characterized by (McMahon et al. 2018). The HOTT mouse is responsive to oxidative stress through oxidants which initiate the activation of the heme oxygenase 1 (*Hmox1*) promoter. The activation of *Hmox1* promoter is followed by the expression of the reporter gene lacZ which in turn can be visualized post-mortem by X-Gal staining. This staining results in the formation of an indigo dye which is visible as blue stained areas within different organs. Correspondingly, the appearance of blue coloration within the HOTT mice tissue reflects oxidative stress.

During the study, mice were group housed in open-top cages at $22 \pm 2^\circ\text{C}$, $55 \pm 10\%$ relative humidity and a 12 h light/dark cycle. Food (RM1, Special Diet Services, Essex, UK) and water were provided *ad libitum*. The study design was structured as follows: Twenty young adult male HOTT mice (6-12 weeks of age) were randomly allocated to groups (5 animals per group) and treated with 2-MCPD at 1, 10 or 100 mg/kg body weight (Toronto Research Chemicals, North York, Canada) by oral gavage daily for 28 days. A control group received PBS (phosphate-buffered saline).

After euthanasia of the animals due to rising concentration of CO_2 , death was additionally confirmed by exsanguination. The kidneys, testes, brain, liver, heart, skeletal muscle and spleen were removed, rinsed with ice-cold sterile PBS, individually weighed and cut. Afterwards the organs were fixed or frozen in liquid nitrogen (-70°C). For kidneys and testes, one organ was fixed with Mirsky's fixative (National Diagnostics, Atlanta, USA) overnight at 4°C and the other one was snap frozen while for heart, spleen, brain and skeletal muscle half

of the organs was fixed and the other half was snap frozen. For half of the left lobe of the liver 10% neutral buffered formalin (VWR International, Lutterworth, Leics, UK) was used and the remainder was snap frozen. After fixation, the tissues were washed in PBS containing 2 mM MgCl_2 before overnight dehydration in 30% sucrose and embedding in Shandon M-1 Embedding matrix (ThermoScientific, Perth, UK) for cryosectioning. For cryosectioning, the fixed organs were cut into 10 μm sections on an OFT5000 cryostat (Bright Instrument Co., Luton, UK) at -20°C .

2.2 Histochemical analysis

LacZ staining

Slides with 10 μm tissue slices were stained as detailed described in (Schultrich et al. 2020). In brief, the tissue sections were rehydrated and stained at 37°C overnight by using an X-Gal staining solution. Afterwards, slides were washed with PBS, counterstained with Nuclear Fast Red and again washed twice with double-distilled water. After dehydrating with 70% and 95% ethanol and incubation in HistoClear, slides were air-dried and coverslips were applied by using DPX mountant.

Densitometric evaluation

The X-Gal-stained organ slices were evaluated using a Zeiss Axio Observer (Carl Zeiss, Jena, Germany). For the quantitative analysis of the X-Gal staining, three images per animal from kidneys, testes, skeletal muscle and heart were used. The quantification of X-Gal positive areas in the cerebellum was performed for three mice per group. Densitometric analysis was done with the software ZEN 2.3 lite blue (Carl Zeiss Microscopy, Thornwood, USA) by using a customized automatic segmentation algorithm. Thereby X-Gal positive pixels (blue colored tissue area) in each image were quantified and normalized to the total area of the image. Accordingly, the area of the X-Gal-stained tissue within each microscopic image in percent was determined.

2.3 Protein extraction and Western blotting

Proteins were extracted from kidneys, testes, brain, skeletal muscle and heart. Therefore the frozen tissues were disrupted and homogenized by a mortar and pestle in liquid nitrogen. 10-45 mg of the frozen tissue powder was transferred to RIPA lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 μ M EGTA, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholic acid, 2% complete protease inhibitor cocktail 1x (Roche, Basel, Switzerland)) and stepwise sonicated (Ultrasonic converters UW 2070, Berlin, Germany). After centrifugation at 20,000 x g for 30 minutes at 4°C, the supernatants containing the extracted proteins were stored at -80°C. Protein determination was conducted by using the Bicinchoninic Acid Kit (Sigma-Aldrich, Munich, Germany) and a microplate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland).

2-Dimensional Western blotting

The 2D gel electrophoresis was carried out according to (Buhrke et al. 2018). For isoelectric focusing, 150 μ g of total protein was separated on 7 cm strips (Immobiline Drystrip pH 3-10 NL, GE Healthcare, Freiburg, Germany) in an Ettan IPGphor IEF separation unit (GE Healthcare, Freiburg, Germany). The following program for protein separation in the first dimension was used: 300 V for 1 h, gradient from 300 to 1000 V within 1 h, gradient from 1000 to 5000 V within 2 h, and finally 5000 V for 1.5 h. For SDS-PAGE, lab-made 12 % polyacrylamide gels (8 x 6 cm) were used, and protein separation was carried out in a electrophoresis chamber (Bio-Rad, Munich, Germany) according to (Laemmli 1970) with an adjusted current of 15 mA per gel. The protein transfer was conducted onto nitrocellulose membranes (Amersham Protran Premium 0.45 NC nitrocellulose Western blotting membrane, GE Healthcare, Freiburg, Germany) using a semi dry blotting system (Amersham TE77 PWR, GE Healthcare, Freiburg, Germany) with 60 mA per gel. The blocking step as well as the dilution of the antibodies was performed with 5% milk (Carl Roth, Karlsruhe, Germany) in TBST (Tris-buffered saline, 0.1% Tween 20). The following antibodies were used: rabbit monoclonal antibody against PARK7/DJ1 (ab76008; Abcam, Cambridge, UK;

1:5000 dilution), and goat-anti-rabbit-IgG-HRP (HAF008, R&D systems, Minneapolis, Canada; 1:2000 dilution). The protein bands were chemiluminescent visualized by using the SuperSignal™ West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific, Waltham, USA) and the VersaDoc MP4000 Imaging System (Bio-Rad, Munich, Germany). Semi quantitative analysis of the protein spot intensities was conducted by using the Image Lab 5.2 software (Bio-Rad, Munich, Germany). The respective spot intensity was related to the total spot intensities of the same blot and presented as relative intensities.

2.4 RNA isolation and quantitative real-time RT-PCR

For total mRNA extraction, 15-30 mg frozen tissue was homogenized and disrupted in liquid nitrogen with a mortar and pestle. Afterwards, total mRNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was determined by using NanoQuant Plate™ together with a microplate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland). cDNA synthesis was carried out using the high capacity cDNA reverse transcription kit (Applied Biosystem/ Life Technologies GmbH, Darmstadt, Germany). cDNA was subjected to quantitative real-time PCR analysis that was performed with the Maxima SYBR Green/Rox qPCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 300 nM of each primer and 1 µl cDNA in a total volume of 10 µl. Stratagene MX3005P (Agilent, Santa Clara, USA) or AriaDx real-time PCR System (Agilent, Santa Clara, USA) were used with the following cycle conditions: initial denaturation at 95°C for 15 min, 40 cycles of denaturation at 95°C for 30 s and annealing and elongation at 60°C for 1 min, final elongation at 60°C for 15 min. At the end of the run, the melting curve was obtained by using dissociation parameters as follows: step 1 at 95 °C for 30 s, step 2 at 60°C for 30 s and step 3 again at 95°C for 30 s. The $\Delta\Delta C_t$ method was used for calculation of the relative gene expression in relation to controls (non-treated, set to a relative gene expression value of 1.0) and with β -actin serving as housekeeping gene. The following primer were used: β -Actin (*Actb*) 5'-GATCATTGCTCCTCCTGAGC-3' (forward) and 5'-CATCGTACTCCTGCTTGCTG-3', glutathione S-transferase P1 (*Gstp1*) 5'-ATATGTCACCCTCATCTACACCA-3' (forward) and

5'-CTGGTCACCCACGATGAAAG-3' (reverse), catalase (*Cat*) 5'-CAATGGCAATTACCCGTCCTG-3' (forward) and 5'-TAGTCCTTGTGAGGCCAAACC-3' (reverse), superoxide dismutase 1 (*Sod1*) 5'-GGGTTCCACGTCCATCAGTAT-3' (forward) and 5'-CCTTTCCAGCAGTCACATTGC-3' (reverse), heme oxygenase 1 (*Hmox1*) 5'-GCAGAACCCAGTCTATGCCC-3' (forward) and 5'-GGCGTGCAAGGGATGATTTC-3' (reverse), glutathione synthetase (*Gss*) 5'-ATGCGGTGGTGCTACTGATT-3' (forward) and 5'-TACGGCACGCTGGTCAAATA-3' (reverse).

Statistics

One Way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison tests was used for statistical analysis. Statistical significance was assumed at $p < 0.05$.

3. Results

Body and organ weights

In order to obtain information about 2-MCPD target organs in mice and about the underlying toxicological mode of action of 2-MCPD, a 28-day feeding study with male HOTT mice was conducted. Mice were treated with either PBS (vehicle control), or with daily doses of 1, 10 or 100 mg 2-MCPD per kg body weight for 28 days. During the study, none of the five animals of each treatment group showed pathological abnormalities or signs of acute toxicity. The body weight of the mice was not significantly affected by 2-MCPD treatment (Fig. 1 a). The organ-to-body weight ratios of kidney, testes, brain, liver, heart and spleen were not substantially altered upon 2-MCPD treatment (Fig. 1 b).

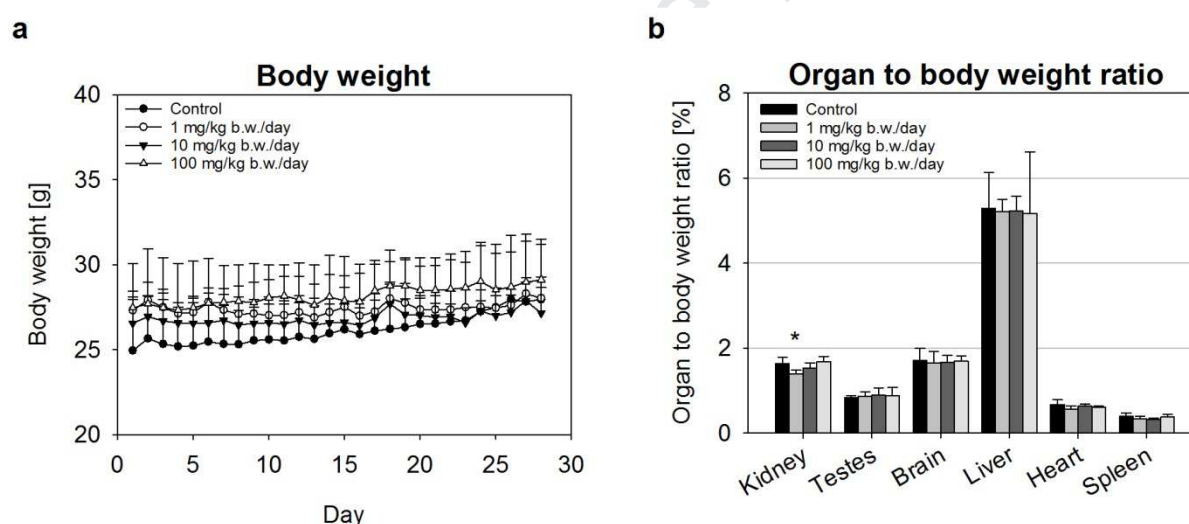


Figure 1 Mean body weights (a) and organ to body weight ratios of kidney, testes, brain, liver, heart and spleen (b) for male HOTT mice treated with 2-MCPD. Five mice per group received either PBS as vehicle control or 1, 10 or 100 mg 2-MCPD per kg body weight for 28 days. The data are expressed as mean + SD (n=5). Statistics by one-way ANOVA followed by Dunnett's multiple comparison test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

A small but statistically significant decrease in relative kidney weight was observed after daily administration of 1 mg 2-MCPD per kg body weight. This decrease, however, was not dose-

dependent, as it was not visible in the middle and high dose groups. Altogether, the data on body and organ weight did not reveal substantial evidence for toxicity of 2-MCPD in mice after repeated dosing up to 100 mg/kg body weight per day.

X-Gal staining of different organs

The reporter gene *lacZ* has been integrated into the genome of the HOTT mouse to visualize compound-induced oxidative stress. The intensity of the blue color thereby correlates with the intensity of oxidative stress (McMahon et al. 2018). Thus, different organs of the 2-MCPD-treated mice were subjected to X-Gal staining. Representative X-Gal stained slices of kidney, testis, cerebellum, heart and skeletal muscle are shown in Fig. 2.

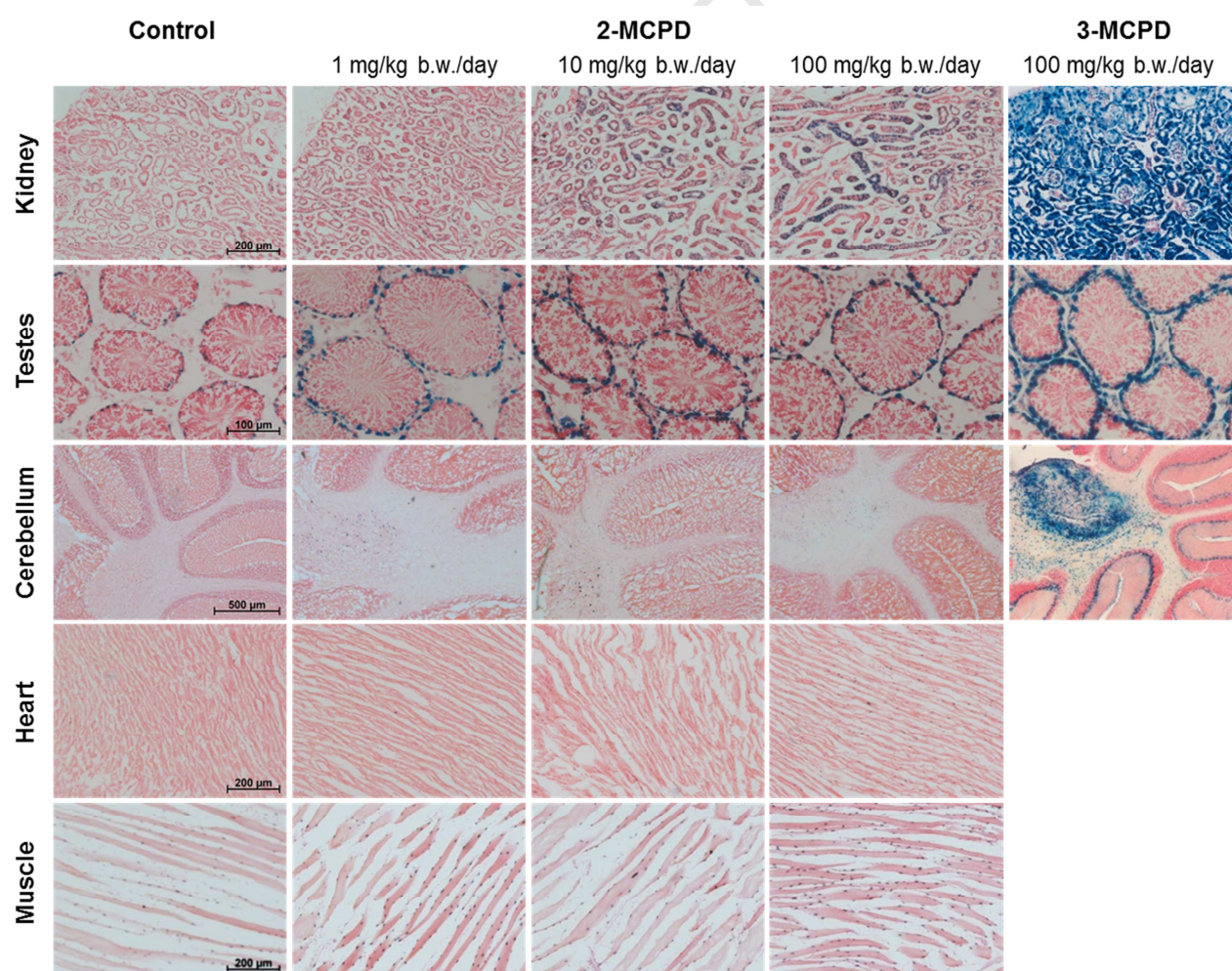


Figure 2 Representative microscopic images of X-Gal-stained slices from kidney, testes, cerebellum, heart and skeletal muscle of HOTT mice. The reporter mice were treated with

PBS, 1, 10 or 100 mg 2-MCPD per kg body weight for 28 days. As positive control, microscopic images of X-Gal stained kidney, testes and cerebellum of HOTT mice treated with 100 mg 3-MCPD per kg body weight over a period of 7 days are depicted (Schultrich et al., 2020).

A dose-dependent increase of X-Gal-positive cells was exclusively visible in the kidney and testes, but not in the other organs. More precisely, the lowest dose of 2-MCPD caused no blue coloration in the kidney whereas there was a dose-dependent increase in blue staining for the higher doses. It has to be noted that the staining was only visible in the renal cortex (Fig. 2). Similar to the kidney, the blue staining of the testes increased with rising 2-MCPD concentrations. The X-Gal-positive cells in the testes were located in the outer part of the organ within the seminiferous tubules (Fig. 2). The overall intensity of staining, however, was only moderate even in the high dose group, as evident from a comparison with X-Gal staining of organs obtained from mice treated with the much more potent 3-MCPD (Fig. 2). For other possible target organs of 2-MCPD toxicity, heart and skeletal muscle, no 2-MCPD-mediated activation of the *Hmox1* promoter and thus no associated blue coloration in these organs were visible. The same applies to the organs brain, liver and spleen (data not shown). The densitometric analysis of the blue-stained organ slices (Fig. 3) underlines the visual results and revealed a significant increase of the blue coloration in the renal cortex and testes after daily treatment with 10 and 100 mg 2-MCPD per kg body weight (Fig 3 a and b). In contrast, the area of X-Gal staining in the microscopic images of the cerebellum, heart and skeletal muscle was far below 2 % and, with exception of the weak increase of staining in the heart in the high dose group, did not reach statistical significance (Fig. 3 c-e).

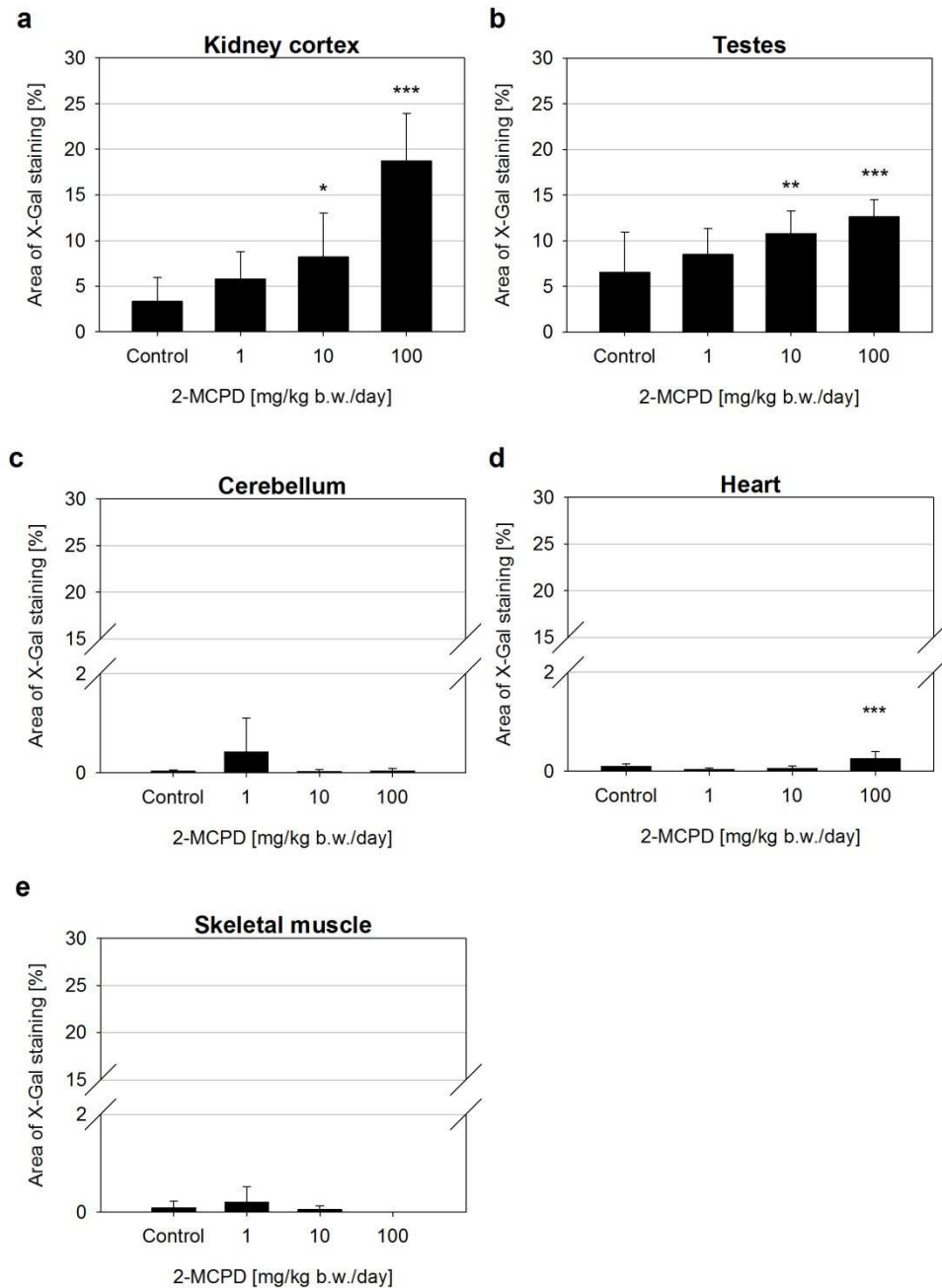


Figure 3 Densitometric analysis of the microscopic images shown in Fig. 2 for X-Gal stained organ slices of HOTT mice. The quantification of the blue-stained area within these images was carried out by using the ZEN lite blue edition software and covers the whole image size. Data that were obtained for kidney (a), testes (b), cerebellum (c), heart (d) and skeletal muscle (e) are expressed as mean + SD (n=5 for all organs except of cerebellum with n=3). Statistics: one-way ANOVA followed by Dunnett's multiple comparison test; * p<0.05; ** p<0.01; *** p<0.001.

Expression of oxidative stress-related genes

To additionally verify the activation of the reporter gene, tissues were also examined at the mRNA level using quantitative real-time PCR. Beside the expression of the endogenous *Hmox1* gene, further genes were investigated which are involved in the antioxidant signaling pathway: *Gstp1*, *Sod1*, *Cat* and *Gss* (Fig. 4) (Ma 2013).

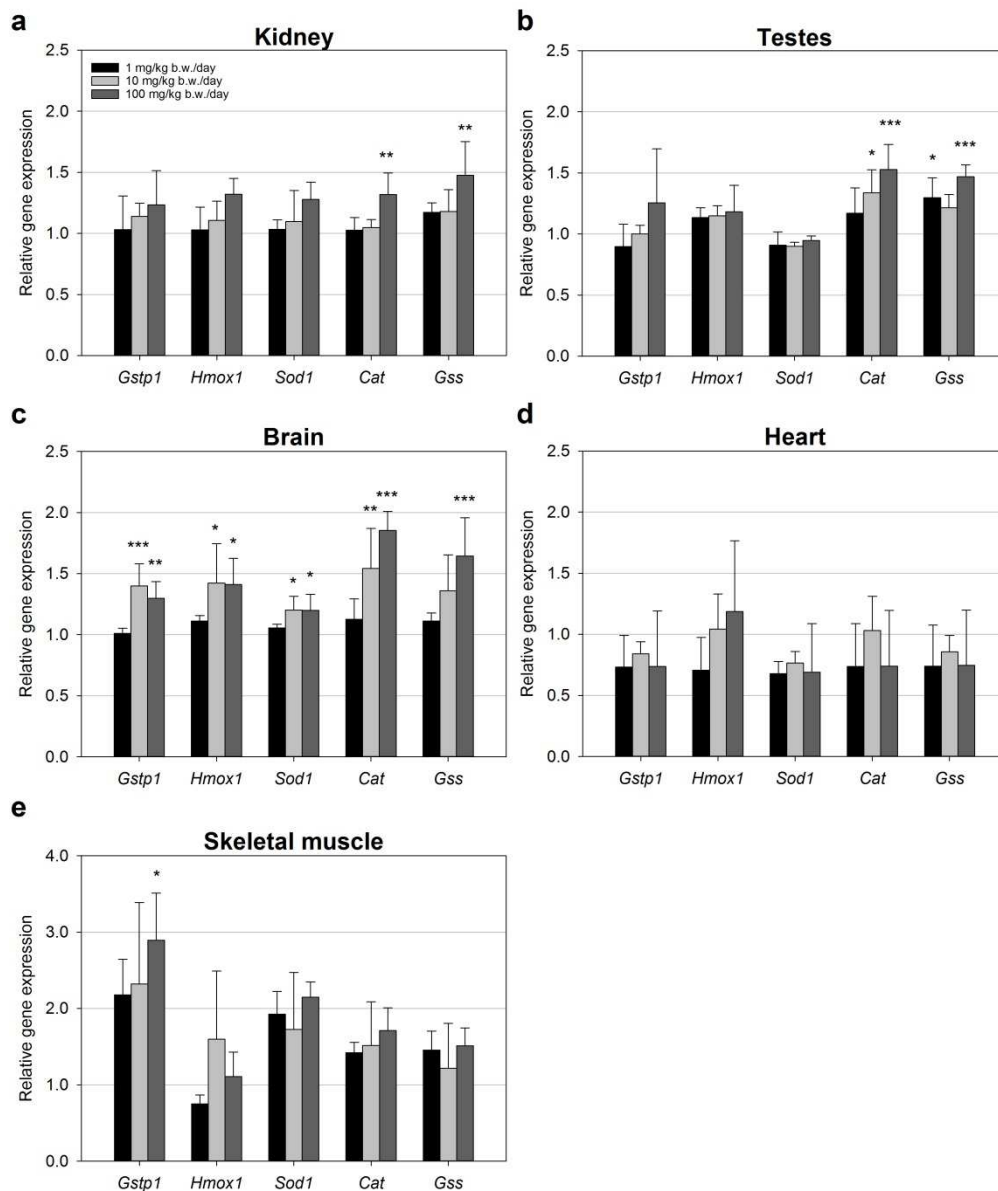


Figure 4 Expression analyses of genes which are involved in the antioxidant signaling pathway. Total mRNA was isolated from kidney (a), testes (b), brain (c), heart (d) and skeletal muscle (e) of male HOTT reporter mice treated with 1, 10 or 100 mg 2-MCPD per kg body weight for 28 days. Relative gene expression of glutathione S-transferase P1 (*Gstp1*),

heme oxygenase 1 (*Hmox1*), superoxide dismutase (*Sod1*), catalase (*Cat*) and glutathione synthetase (*Gss*) is expressed relative to controls (non-treated, assigned value of 1.0) and in relation to β -actin (*Actb*) as housekeeping gene. The relative gene expression was calculated using the $\Delta\Delta C_t$ method and expressed as mean + SD (n=5). Statistics: by one-way ANOVA followed by Dunnett's multiple comparison test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Gene expression of *Cat* and *Gss* was slightly but significantly increased upon 2-MCPD treatment in kidney, testes and brain at higher doses, whereas almost no changes in gene expression were detected in heart and skeletal muscle (Fig. 4 a-d). The majority of changes in gene expression was detected in brain, with a significant increase in all investigated genes after oral administration of 10 and 100 mg 2-MCPD per kg b.w. per day (Fig. 4 c). In the skeletal muscle, a dose-dependent increase in *Gstp1* expression was observed, which was statistically significant in the high-dose group (Fig. 4 e). However, the observed alterations did not exceed a level of 3-fold which is considered to be of minor relevance in terms of oxidative stress response at the transcript level.

Oxidation of DJ-1/PARK7

Under oxidative stress conditions, the redox-responsive protein DJ-1 has been shown to be oxidized at a particular cysteine residue, for example by 3-MCPD (Buhrke et al. 2018). Thus DJ-1 is considered to be multi-tissue effect marker for oxidative stress. In order to examine the effect of 2-MCPD on the redox status of DJ-1, 2D Western blotting analyses were performed. In all organs at least four different DJ-1 spots (named A to D) were detected, of which the D spot was the most prominent one (Fig. 5 a).

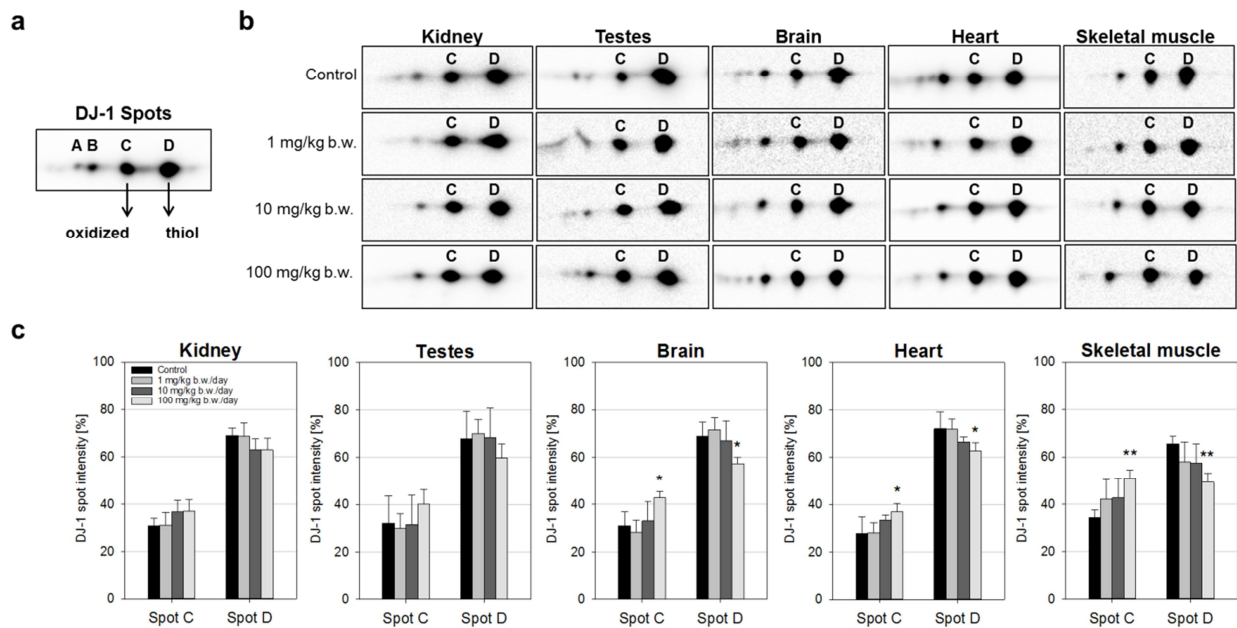


Figure 5 Western blot analysis of the stress response protein DJ-1 after 2D gel electrophoresis. Protein extracts of different organs from HOTT mice treated with PBS (control), 1, 10 or 100 mg 2-MCPD per kg body weight for 28 days were separated by 2D gel electrophoresis. (a) Representative image of a 2D Western blot obtained with protein extract from mouse heart in order to demonstrate four DJ-1 isoforms named A-D. (b) Representative 2D western blot images obtained with protein extracts from kidney, testes, brain, heart and skeletal muscle of the HOTT mice for all treatment groups. (c) Densitometric analysis of the 2D Western blot images for the most prominent DJ-1 isoforms (C and D) expressed as mean + SD (n=5). Statistical analysis was carried out by one-way ANOVA followed by Dunnett's multiple comparison test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

A previous study has shown that the C spot corresponds to the oxidized form of DJ-1 (non-functional), whereas the D spot represents the non-oxidized form (functional) (Buhrke et al. 2018). Representative 2D Western blot images showing the different DJ-1 spots upon 2-MCPD treatment in kidney, testis, brain, heart and skeletal muscle are shown in Fig. 5 b. By comparing the intensity of the C and D spots from the control groups to those obtained after 2-MCPD treatment, no strong alteration of the C and D spots was visible in the investigated organs. A minor shift from the D spot to the more acidic C spot was observed in kidney and

testis as well as in brain and heart in mice treated with the highest 2-MCPD dose. These results were further underlined by a semi-quantitative analysis of the C and the D spot intensities (Fig. 5 c). The low and middle dose of 2-MCPD did not result in a significant shift of the two protein spots in any organ, whereas in brain, skeletal muscle and heart the highest 2-MCPD dose resulted in a modest but significant decrease of the D spot with a corresponding increase of the C spot. This corresponds to a loss-of-function of DJ-1 via protein oxidation. However, it should be noted that this effect was only minor, thus not pointing towards pronounced oxidative stress induced by 2-MCPD.

4. Discussion

Little is known about the toxicity of 2-MCPD and the underlying molecular mechanisms. In the present study, male HOTT reporter mice were employed to examine to which extent 2-MCPD exposure is associated with the induction of oxidative stress. During the study, animals orally received 1, 10 or 100 mg/kg body weight per day 2-MCPD over a period of 28 days, or PBS as a control. The animals showed no signs of acute toxicity after treatment and were in a healthy condition during the entire study. In a previous animal experiment with analogous study design using 3-MCPD, mice of the high dose group (100 mg 3-MCPD per kg body weight per day) showed a severe decrease in body weight of more than 20% within seven days (Schultrich et al. 2020). This clearly shows that a daily oral application of 100 mg 3-MCPD per kg body weight caused acute toxic effects in male HOTT mice, whereas identical doses of 2-MCPD did not lead to any clinical symptoms or significant toxicity, indicating that, at least in mice, 2-MCPD is less toxic than 3-MCPD. This is in line with a 28-day oral subacute toxicity study with male Wistar rats investigating the effects of 3-MCPD and 2-MCPD at the molecular level: transcriptomic analyses revealed that 3-MCPD affected numerous genes in kidney, and to a lesser extent also gene expression in liver and testes, whereas 2-MCPD showed overall less changes as compared to 3-MCPD (Buhrke et al. 2017).

Kidney and testes have been identified as the main target organs for 3-MCPD toxicity in numerous studies, whereas only very limited data are available for 2-MCPD. According to an EFSA report, 28-day oral administration of 16 and 30 mg 2-MCPD per kg body weight induced lesions in striated muscle and lysis of myocytes with most pronounced effects in the myocardium. The same 2-MCPD concentrations also caused increased kidney weights and renal tubule changes (EFSA 2016). Based on this study, skeletal muscle, heart and kidney seemed to be the main target organs for 2-MCPD toxicity in rats. In line with transcriptomic data from (Buhrke et al. 2017), proteomic analyses of tissues from the same rat study revealed that 3-MCPD mainly affected protein expression in the kidney, contrary to 2-MCPD, where most alterations were observed for the heart proteome (Frenzel et al. 2018a; Schultrich et al. 2017). Considering these studies, it can be concluded that despite the structural similarity of 3-MCPD and 2-MCPD, different target organs are addressed. For 2-MCPD, the main target organs appear to be heart, kidney and skeletal muscle. Indications that both substances not only have different target organs, but also different mechanisms of action, were also shown by the 2D-GE/MS-based proteomic study with the main focus on rat heart: this study revealed a higher number of differentially expressed proteins upon 2-MCPD treatment than upon 3-MCPD treatment, corroborating the finding that of myocardium is primarily a target of 2-MCPD, but not of 3-MCPD toxicity. Striking protein deregulation was observed for example for the mitochondrial Cytochrome b-c1 complex subunit Rieske which is an important protein in the respiratory chain (complex III) in order to generate energy (Smith et al. 2012). Interestingly, this enzyme was strongly deregulated upon 2-MCPD treatment, but not after 3-MCPD treatment. Deregulation of this protein could therefore be associated with altered mitochondrial activity and energy formation, which could be due to compensation of the heart. Such compensation is assumed, for example, in hypertrophic hearts (Rosca and Hoppel 2010). Heart hypertrophy was a possible toxic effect, which was bioinformatically predicted in the rat heart proteome study. However, neither in the rat proteome study nor in the present mouse study the heart-to-body weight was increased (Schultrich et al. 2017; Schultrich et al. 2020). Although the specific mechanism of action of

2-MCPD is unclear, the data show that 2-MCPD not only targets other organs compared to 3-MCPD, but also triggers different toxic mechanisms at the molecular level. This appears especially plausible since (i) the chemical structures of 2-MCPD and 3-MCPD are likely to lead to different series of metabolites and since (ii) 3-MCPD-specific metabolites have been implicated in the toxicity of the latter compound (Jones 1983; Lynch et al. 1998).

In addition to the identification of potential target organs, the abovementioned transcriptome and proteome analyses also provided information on the putative molecular mechanisms of action of both substances. In this context, the omics data pointed to a substance-induced induction of oxidative stress upon 3-MCPD treatment and, to a lesser extent, upon 2-MCPD treatment (Buhrke et al. 2017; Frenzel et al. 2018a; Frenzel et al. 2018b; Schultrich et al. 2017). With the focus on oxidative stress response, a 28-day oral feeding study showed that orally administered 3-MCPD led to an activation of the ROS-dependent *Hmox1* promoter, especially in kidneys, testes and specific brain areas such as midbrain, cerebellum and pons (Schultrich et al. 2020). In contrast to the 3-MCPD study, the present study with 2-MCPD revealed a significantly lower *Hmox1* promoter activation in kidneys and testes, and no induction of the promoter was observed in brain, heart and skeletal muscle. The lack of corresponding dose-dependent effects at the organ weight and/or pathology level indicates that the observed molecular effects at the level of *Hmox1* promoter activation have occurred in a dose range not yet exerting clear-cut adverse effects, in line with the assumption that molecular effects are expected to be detectable with greater sensitivity than adverse outcomes at the organ level. Our data allow to conclude that 2-MCPD hardly induced oxidative stress in mice at the administered doses, which underlines the different mode of action when directly compared to the more potent oxidative stress inducer 3-MCPD. In addition to *Hmox1* promoter activation as a marker for oxidative stress, expression analyses of Nrf2-dependent genes which are involved in the antioxidant response signaling were performed (Ma 2013; Tonelli et al. 2018). The overall weak gene expression changes were consistent with the observed low *Hmox1* promoter activation and finally support the notion that 2-MCPD does not remarkably activate the Nrf2-dependent oxidative stress response.

Beside expression analysis at the mRNA level, the post-translational modification of a specific protein named DJ-1 (*Park7*) was examined. The protein DJ-1 is considered as a marker for oxidative stress as a specific cysteine residue (Cys106) is of particular importance for sensing and attenuating oxidative stress. Under oxidative stress conditions, Cys106 of human DJ-1 is oxidized via sulfenic acid and sulfinic acid and finally to the irreversible oxidation to sulfonic acid. The irreversible oxidation of Cys106 to sulfonic acid is accompanied by a loss of function of the protein (Wilson 2011). It has been shown that repeated oral doses of 10 mg 3-MCPD induced irreversible oxidation of DJ-1 in rat kidney, liver and testes, indicative of oxidative conditions. The same doses of 2-MCPD, on the other hand, led to a remarkably lower level of DJ-1 oxidation only in rat heart and kidney (Buhrke et al. 2018; Schultrich et al. 2017). The above results obtained with rats are partially consistent with those gained with mice. Similarly to the rat, a marked oxidation of DJ-1 was also detected in mouse kidney and testes, which already occurred after repeated administration of 1 mg 3-MCPD and which was additionally observed in mouse brain (Schultrich et al. 2020). In contrast to 3-MCPD, the present study demonstrates that 2-MCPD hardly affects the oxidation status of DJ-1 in various organs. Exclusively after treatment with the highest dose of 2-MCPD, a significant but nonetheless still only modest increase of oxidized DJ-1 was visible in mouse brain, heart and skeletal muscle. With respect to the situation in brain, the slight changes in DJ-1 together with the moderate up-regulation of oxidative stress response genes might indicate possible effects of 2-MCPD also on the central nervous system. These molecular observations, however, were not correlated with organ weight or histopathological observations indicating an adverse effect, and studies with longer duration and/or even higher doses would be needed to clarify the potential of 2-MCPD to induce toxic effects in mouse brain. Overall, 2-MCPD appears to have a considerably lower potential to exert oxidative stress than 3-MCPD, suggesting different molecular toxicity mechanisms of both substances despite their similar chemical structure.

In a 28-day toxicity study in rats, 2 mg 2-MCPD per kg body weight per day was recorded as no observed adverse effect level, whereas 16 mg 2-MCPD caused histopathological changes

(EFSA 2016). A 28-day study with 3-MCPD in mice at dose levels of 1, 10 and 100 mg 2-MCPD per kg body weight yielded toxic effects at the highest administered dose, and molecular effects related to oxidative stress induction down to the lowest dose used. We therefore decided to study the impact of 1, 10 and 100 mg 2-MCPD per kg body weight on oxidative stress in mice in the present study, in order to allow for a direct comparability to 3-MCPD data in the same mouse strain. Nevertheless, it is important to note that the applied 2-MCPD doses in the present mouse study are orders of magnitude above those that can be achieved by human chronic intake via diet.

Considering highly exposed consumers (95th percentile) the maximum value for the chronic exposure to 2-MCPD is 0.4 µg/kg body weight per day for adults (EFSA 2016). This value is lower by a factor of 2500 compared to the lowest dose of 1 mg/kg used in the present mouse study, where no adverse effects occurred. It is therefore unlikely that these concentrations can be reached via human food intake.

In conclusion, the results of the present study reveal i) no acute toxicity of up to 100 mg 2-MCPD per kg body weight per day in mice, ii) an only mild induction of oxidative stress by 2-MCPD in brain, heart and skeletal muscle (as shown by DJ-1 oxidation) which is much less pronounced than the effects exerted by identical doses of 3-MCPD. Thus, 2-MCPD had a considerably lower impact on oxidative stress compared to 3-MCPD indicating that the molecular toxicity mechanisms of 2-MCPD and 3-MCPD are probably different. This underlines the necessity for individual assessment of the toxicity of these two closely related substances.

Acknowledgments

We thank Christine Meckert for helpful technical assistance. Tanya Frangova is thanked for help with animal work, Aileen McLaren for tissue collection and Cheryl Wood for tissue sectioning and staining. This project was financed by the German Federal Institute for Risk Assessment (project 1322-660). The HOTT mouse was developed within the European Research Council Advanced Investigator Award under the number 294533.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in this study involving animals were in accordance with the ethical standards of the Welfare and Ethical use of Animals Committee of the University of Dundee (permit number ND01/09/16).

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Highlights:

- Up to 100 mg 2-MCPD per kg body weight per day caused no acute toxicity in male mice
- Only minor effects of 2-MCPD on the induction of oxidative stress in mice
- Different modes of action of 2-MCPD and 3-MCPD with regard to target organs and oxidative stress

CRediT author statement

Katharina Schulrich: investigation, writing – original draft preparation; **Colin Henderson:** methodology, resources; **Thorsten Buhrke:** funding acquisition, supervision, conceptualization, writing – review & editing; **Albert Braeuning:** conceptualization, writing – review & editing

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: